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Experiments completed in the past year were designed to characterize the role of Vascular Endothelial Growth Factor (VEGF) and its receptors (VEGFr) in the progression of breast cancer. VEGF and VEGFr are known to be involved in tumor angiogenesis and many other vascular proliferative disorders. Since VEGF and VEGFr are intimately involved in generating the vascular invasion in tumors, they are likely to be important prognostic indicators. VEGF mRNA is alternatively spliced into 4 isoforms, while there are 2 high affinity receptors for VEGF. The relative roles and involvements of each isoform and receptor in cancer is unknown. Since VEGF can be regulated by estrogen, we have grown tumors (human MDA-MB-231 and murine MXT-OVEX) in ovariectomized mice implanted with either estradiol or placebo pellets. We proposed to study the effect of estrogen on the expression of the VEGF isoforms and receptors in the normal mammary gland and in breast cancers in ovariectomized animals. We have characterized the expression of all 4 VEGF isoforms and the 2 receptors using PCR and competitive PCR. Our most useful advance has been the development of a competitive PCR method to quantitatively detect both Flk and Flt in tissues (breast cancer). This will facilitate and likely speed up progress of Task 7 (originally slated to be completed in year 4), the development of an assay to measure vascular invasion in breast cancer.

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(5) INTRODUCTION

Significant progress has been made in the detection and treatment of breast cancer in the past 10 years, however, breast cancer is still one of the leading causes of death in women (1,2). There are 150,000 new breast cancer diagnoses each year (3). Surgery alone results in cure or long-term remission in only 50% of the cases and disease recurrence is an astounding 80% within 10 years in node-positive cases (4). Presently, many tumor characteristics are used as indicators of the future clinical course of women with breast cancer, however, structural or functional components of tumor growth that are associated with aggressiveness are yet unknown (5). Obviously, more sensitive methods for early detection and a better understanding of the etiology and pathogenesis of breast cancer are necessary to combat this disease.

Tumor growth and metastatic spread of the primary tumor are generally accepted to be angiogenesis dependent. The ingrowth of the surrounding vasculature to supply the tumor with nutrients and provide a conduit in which tumor cells can travel to distant sites is absolutely necessary for tumor growth beyond 2 mm in diameter and for the development of distant metastases (6-12). Numerous retrospective and prospective studies have demonstrated that angiogenesis (high microvessel density) in clinical breast cancer is positively correlated with increased recurrence, metastasis, and mortality in both node positive and node negative patients and has proven to be one of the best prognostic indicators presently available (13-15).

The mechanisms by which the host vasculature invades tumors are numerous and complicated. However, the primary and critical event is the release of an angiogenic factor from the tumor cells or the surrounding extracellular matrix (16,17). The search for tumor-derived angiogenic growth factors has been an area of intense effort over the past 20 years. Many angiogenic factors have been described, but only one known factor, vascular endothelial growth factor (VEGF, also known as vascular permeability factor, VPF)(18,19), acceptably satisfies the many criteria to be considered an angiogenic factor outside of experimental models. Originally identified as a tumor-secreted protein which increased vascular permeability *in vivo*, VEGF is also angiogenic *in vivo* (20-22). VEGF is a homodimeric protein of ~46 kD produced by many cell types, including a variety of tumors,

folliculostellate cells, macrophages, and possibly podocytes or capsular epithelial cells in the renal glomeruli among others (23). VEGF is highly conserved with 88-98% homology among murine, rat, bovine, and human sequences (24-26). Interestingly, the VEGF mRNA is alternatively spliced into 4 isoforms of 121, 165, 189, and 206 amino acids (120, 164, 188, and 205 aa in mice) (27,28). Although all 4 isoforms have a signal peptide sequence, only the two shorter proteins are believed to be secreted while the longer forms remain associated to the cell membrane or extracellular matrix. Yet, the 121 aa isoform does not bind heparin well and the 165 aa isoform does (29). All 4 species can cause endothelial mitogenesis and increase vascular permeability in experimental systems, but functional differences, relative potencies, and tissue distribution *in situ* are not well established. Since there are significant biochemical differences among the 4 isoforms (secretion, heparin binding), it is reasonable to hypothesize that they will have different functions and/or be produced by different cells or in different locations. In fact, there is evidence that a non-soluble form of VEGF is responsible for vascular patterning in quail development, such as $VEGF_{189}$ (30). Unlike the other putative tumor angiogenesis factors, bFGF and PD-ECGF, VEGF is specific for endothelial cells. It does not increase mitogenesis in any other cell type and its receptor has only been directly localized to endothelial cells (31,32).

There are two high affinity receptors identified for VEGF, fms-like tyrosine kinase (Flt-1) and fetal liver kinase (Flk-1) (both homodimers of 180 and 205 kD, respectively) (33,34). As their names suggest, both are receptor tyrosine kinases and appear to be exclusively expressed on endothelial cells. Although the receptors are related to the PDGF receptor, they represent a new class of receptor tyrosine kinases since their extracellular domain contains 7 immunoglobulin repeats rather than the standard 5 (33). The VEGF receptor mRNA has been detected in the endothelium of many tissues but at different levels of expression, with proliferating vascular endothelial cells (due to either pathology or normal development) expressing the highest levels (35,36). In fact, except for some fenestrated endothelium in the kidney glomerulus and choroid plexus, non-proliferating vascular endothelium does not express detectable levels of either receptor (36). The receptors are the first proteins expressed on endothelium in development and are critically necessary for animal development as evidenced by the fact that the receptor knockouts are embryonic lethal (37,38). However, no studies have adequately quantitated

the level of receptor expression in tumor vasculature. As with the 4 isoforms of VEGF, differences in function and *in vivo* distribution between the 2 receptors in normal or cancer tissue are unknown.

Tremendous research interest has developed in VEGF and its receptor, in part, due to their ubiquitous presence at times of vascular proliferation and unique regulation. Both the growth factor and its receptors have been shown to be intimately involved in developmental vasculogenesis, demonstrating spatial and temporal regulation throughout the vascular development of the embryo (39). Moreover, VEGF is believed to be involved in, if not responsible for, the angiogenesis during wound healing (40) and numerous pathologies, which include diabetic retinopathy (41), rheumatoid arthritis (42), chronic inflammation (43), psoriasis (44), as well as, numerous malignancies (35,45-47), including breast cancer (14,48). In short, in every case where angiogenesis is a prevalent pathological characteristic, VEGF has been found.

Many markers are currently used as prognostic indicators in breast cancer. The "perfect" prognostic indicator has been described as one which is functionally involved in the generation and progression of the disease (49). Studies on breast cancer and its progression (pathogenesis) have resulted in the identification of numerous prognostic indicators. Traditional prognostic indicators have included host factors such as age, menopausal status, and nodal involvement. As the mechanisms of cancer progression and tumor growth became elucidated, new prognostic indicators began to focus on general biological attributes of cancer cells, such as proliferation markers, proteases, growth factors, and oncogenes (49,50). Much of the data on the prognostic value of these markers has been conflicting, but many of these markers have proven to be moderate to poor indicators of either disease free survival or overall survival (49). Tumor angiogenesis, measured by counting vessels immunohistochemically stained for CD31 or factor VIII antigen, has proven to be an independent and highly significant prognostic indicator in predicting overall survival and relapse-free survival, being as good as or better than other commonly used indicators (51-54). Unfortunately, these currently used markers are not "perfect". CD31, although highly expressed on endothelium, is found on other cells (e.g. platelets), has no known function in angiogenesis, and is not specific for neovasculature. Additionally, factor VIII antigen is variably expressed on

microvasculature, often not identifying smaller vessels. Furthermore, counting immunohistochemically stained microvessels is a qualitative method subject to observer variations. The development of a quantitative method of a tumor vascular specific marker which could not be biased by individual observers would be superior. It is possible that the VEGF receptors are excellent prognostic indicators of microvascular density.

In the past year we have undertaken studies to characterize the involvement of all four VEGF isoforms and two receptors in breast cancer using PCR. Additionally we have developed a competitive PCR assay to quantitatively measure the expression of both VEGF receptors, Flk and Flt, in breast cancer. Continued experiments will determine whether measurement of the VEGF receptors is representative of the microvascular density and therefore might replace the qualitative and laborious method of counting immunohistochemically stained microvessels.

(6) BODY OF PROPOSAL

Methods

Tumor Generation

Female mice were ovariectomized (OVX) and implanted with either an Estradiol pellet or placebo pellet (Innovative Technologies Research, Toledo, OH). Murine (MXT-OVEX) and human breast carcinoma (MDA-MB-231, MCF-7) cells were injected into the 3rd mammary fat pad of ovariectomized mice. Once tumors reached ~5-8 mm diameter, the animals were sacrificed, the tumors were removed and processed for microscopy and biochemical analysis. Additionally, normal mammary tissue from ovariectomized animals with estradiol pellets or placebo pellets was harvested.

RNA and Protein Isolation

RNA and protein were isolated using the Trizol Reagent according to manufacturer's instruction (Gibco BRL, Gaithersburg, MD). Total RNA was treated with

DNase, phenol:chloroform extracted, ethanol precipitated and 2 μg were reverse transcribed to generate cDNA for PCR. Proteins were used in Western blotting.

VEGF PCR

The following primers were used for PCR generation of the VEGF isoforms: upper- 5'-CACCAAAGCCAGCACATAG-3' and lower- 5'-CCGCCTTGGCTTGTCACATC-3'. These primers were designed to pull out all VEGF isoforms from either murine or human tissues (Fig. 1a). The following PCR reaction was used: 1 μl RT-cDNA, 0.4 μM upper and lower primer, 5 μl Pfu PCR buffer, 2.5 μl dNTP (2.5 mM ea.), 38 μl H2O and 1 μl Pfu (Stratagene, La Jolla, CA) using the following method (95°C 5 min; 95°C 30 sec, 54°C 1 min, 72°C 2 min for 30 cycles; 72°C 10 min). PCR products were separated on a 3%/1% Nu-Sieve/agarose gel, imaged and analyzed using a digital gel documentation system. Densitometric analysis of the isoform bands and a DNA Mass Ladder (Gibco BRL) was used to determine data. Bars represent average ± SD of tissue taken from 2-6 animals.

Generation of templates for competitive PCR

Flk and Flt templates were RT-PCR cloned from murine fetal total RNA. RNA was isolated from murine Balb/c fetuses using the Trizol reagent. RNA (5 μg) was reverse transcribed using Superscript II and random primers according to the manufacturer's instruction. Templates for the N-terminal region of mu Flk (corresponding to aa 56-169) and mu Flt (corresponding to aa 186-327) were PCR cloned using the following primers (Flk: upper: 5'-GACCTGGACTGGCTTTGG-3'; lower: 5'-TCTCTTTTCTGGATACCT-3') (Flt: upper: 5'-ACATGGGACAGTAGGAGA-3'; lower: 5'-ACGGAGGTGTTGAAAGAC-3'). Primers were designed using Oligo 4.0 (National Biosciences Inc. Plymouth, MN). The following PCR reaction was used: 1 μl RT-cDNA, 0.4 μM upper and lower primer, 5 μl Pfu PCR buffer, 2.5 μl dNTP (2.5 mM ea.), 38 μl H2O and 1 μl Pfu using the following method (95°C 5 min; 95°C 30 sec, 58°C 1 min, 72°C 2 min for 30 cycles; 72°C 10 min). Clones were verified by restriction digest mapping and sequencing.

Competitive templates for Flk and Flt were generated by using AatII and AflII, respectively, which cut once in the insert but not in the vector. Briefly, 5 μg pET28:Flk was cut with AatII and blunted with T4 DNA polymerase, while pET28:Flt was blunted with Kleenow following restriction digestion with AflII. A 100 bp insert was cut from the pET vector (no insert) and blunted with Kleenow. The blunt ended vectors and 100 bp insert were gel purified prior to overnight in-gel ligation. This generated competitive templates which were 100 bp larger but with identical primer sites to the wt template.

Competitive PCR

PCR reactions were as follows: 1 μl tumor RT-cDNA, 0.4 μM upper and lower primer, 5 μl Pfu PCR buffer, 2.5 μl dNTP (2.5 mM ea.), 20 μCi ^{32}P-dCTP, 28 μl H2O, 1 μl Pfu and 10 μl of competitive template (0-1000 fg) using the following method (95°C 5 min; 95°C 30 sec, 58°C 1 min, 72°C 2 min for 40 cycles; 72°C 10 min). Master mixes of the PCR reaction were made and aliquoted to which 10 μl of each template was added. Reactions (20 μl) were separated on a 6% acrylamide gel made with Tris Borate EDTA buffer, dried and exposed to a phosphorimager screen. Sample and template bands were quantitated using ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA). Values were analyzed and plots of template amount versus template/sample ratio were generated to calculate the amount of sample (55-57). Bars represent the average ± SEM of 2-4 samples. Absolute values (fg receptor) obtained varied less than 15% among replicate experiments.

Electron Microscopy

Animals were sacrificed, the mammary gland or tumor was removed and immediately placed in 3% formaldehyde. Smaller pieces of tissue were then fixed in glutaraldehyde and processed as previously described for electron microscopy (58). Thin (50-55 nm) sections were cut (Reichert-Jung Ultracut E; Vienna, Austria), picked up on copper grids, and stained with uranyl acetate and lead citrate prior to examination and photography (Phillips CM10 electron microscope at 80 kV).

Results

We believe we have accomplished more than what we had set out to do in the first year. However, there were difficulties which shall be described first. Namely, our inability to grow the estrogen sensitive murine carcinoma, MXT, *in vivo* . We do not believe that this will deter us from obtaining our stated goals nor inhibit our efforts to progress with our research. We have simply modified our focus to study the effects of estrogen on the remaining tumors. Additionally, we had hoped to quantitatively analyze both VEGF and VEGF receptor protein. Unfortunately, the antibodies which are commercially available to either the growth factor or the receptors have not proven to be satisfactory. Antibodies to VEGF are primarily made against the human 165 aa isoform and often do not recognize the other isoforms. This has proven to be the case with antibodies obtained from Santa Cruz Laboratories and Chemicon. We have found an antibody which recognizes all three isoforms tested (121, 165, & 189) and cross-reacts with murine VEGF, but the antibody has not proven sensitive enough to be useful in our studies. We therefore used PCR and designed the primers so that they would recognize murine or human VEGF equally and distinguish among the isoforms. Commercial antibodies to the VEGF receptors are notoriously poor. We had generated antibodies in rabbits which were somewhat useful for blotting endothelial cell lysates but not tissue lysates. Interestingly, our antibodies were almost as sensitive as commercially available antibodies (Santa Cruz Laboratories). We have tried immunoblotting and immunoprecipitation with less than satisfactory results. However, we have generated new antibodies to both Flk and Flt in chickens and they have proven better than commercially available antibodies. The reason for immunizing chickens was due to reports describing better Ab generation against highly conserved antigens, such as the VEGF receptors. These antibodies are presently being tested for immunohistochemical staining of mammary and breast cancer tissue. The commercially available antibodies to Flk and Flt do not work satisfactorily in immunohistochemistry.

The Statement of Work for the first year included three tasks, growth of the tumors, analysis of the VEGF isoforms in the tumors, and analysis of the receptors in the tumors.

VEGF Isoforms

PCR was used to determine which isoforms were present in mammary tissue, MXT-OVEX and MDA-MB-231 tumors with or without estrogen (Fig. 1b). The 205 aa isoform was never observed in any sample. Densitometric analysis revealed that the 165 aa isoform had the highest expression levels in all samples, but there were significant differences found in the expression levels of the 121 and 189 aa isoforms between tumor samples and normal mammary tissues (Fig. 1c). Moreover, estrogen increased the expression of VEGF in the normal mammary, but had little effect on the MDA-MB-231 and MXT-OVEX tumors. Interestingly, not all the isoforms were upregulated with estrogen presence. The only significant increase in expression with estrogen was noticed with the 165 and 189 isoforms in the normal mammary tissue. Although there was a dramatic upregulation of VEGF expression in the normal mammary, the levels were always highest in the tumors with the exception of the 189 isoform which was lower in the tumors than in the normal mammary with estrogen (Fig. 1c).

VEGF Receptors

A schematic of the competitive templates is shown in Fig. 2a. Although the wild type (wt) PCR fragment for either Flk or Flt are 100 bp shorter than the competitive template, both template and wt have identical priming sites which insures that the efficiency of PCR for template and wt is identical. Data are analyzed by comparing the ratio of template versus sample signal for a given template input (Fig. 2b). Similar to results with VEGF, estrogen increases the expression of Flk in the normal mammary tissue (Fig. 2c.). The expression of Flk and Flt in MDA-MB-231 tumors is slightly increased in response to estrogen. Whereas, Flk receptor expression in MXTOVEX tumors does not appear to be different between estrogen positive or negative mice and Flt appears to be decreased substantially with estrogen presence. Repeated experiments on Flt expression must be completed since these data are taken from only 2 animals.

Mammary Vascular Morphology

Although these tasks were to be completed in the second year, the samples were obtained at the same time RNA and protein was isolated. We have only initiated these studies but have observed some very interesting modifications in the normal mammary microvasculature with estrogen. Normal mammary gland vessels from ovariectomized mice with placebo pellets are not unlike virgin intact mammary gland vessels, in that, the endothelium is continuous with a large number of caveolae (Fig. 3a). However, the mammary gland vascular endothelium in OVX animals is often modified to become fenestrated (Fig. 3b) and have fused, enlarged caveolae (Fig. 3c). These morphological modifications may be due to the increased VEGF expressed in the mammary gland in response to estrogen (Fig. 1c). Vascular endothelium in MXT-OVEX, MDA-MB-231, and MCF-7 tumors is often fenestrated and has clustered, fused caveolae regardless of whether estrogen is present or not (data not shown).

Discussion

The original proposal was designed to examine 4 tumors: 2 estrogen sensitive (MCF-7 & MXT) and 2 estrogen insensitive (MDA-MB-231 & MXT-OVEX). One setback was the inability to grow the MXT tumors despite repeatedly obtaining the cells from American Type Culture Collection (ATCC). They also did not grow well in culture. Additionally, the MCF-7 tumor has grown extremely slowly and we have been unable to generate more than three usable tumors. Some of this may be due to the design of the experiments, in that, we utilize tumors which have been maintained *in vivo* for at least 3 passages. It may be that the MCF-7 tumor cells are better grown in culture and then transplanted. We have therefore decided to focus on the effects of estrogen on the MXT-OVEX and MDA-MB-231 tumors since the vasculature of the host tissue which supplies these tumors (the mammary gland) has shown to be significantly affected by estrogen. However, we shall begin inoculating the MCF-7 tumors solely from tissue culture and may attempt to grow the MXT tumors again. However, we do not believe the lack of these 2 tumors will compromise our ability to adequately address the specific aims in the grant, generate meaningful data which will increase our understanding of VEGF and its receptors

in breast cancer, or design a sensitive assay to measure a prognostic indicator which may have real value to women with breast cancer.

VEGF expression has been shown to be hormonally regulated in the uterus and upregulated in response to estradiol (59,60). It was therefore not surprising to find that VEGF expression was increased in mammary tissue in response to estrogen. However, the finding that not all isoforms are increased is intriguing. This is the first observation to our knowledge that the isoforms can be differentially regulated. This, of course, presents the question of how does this occur which may be addressed in future studies. Since the different isoforms are alternatively spliced, it is conceivable that there is a splicing factor which is hormonally activated in the mammary gland. It is understandable that the tumors generate more VEGF than the mammary glands even in the presence of estrogen since the tumor mass is much larger and the tumor cells necessitate the production of VEGF and a neovasculature to survive. An interesting finding was the overall lower amount of VEGF 189 in tumors than in mammary tissue with estrogen. Whether this increase is indicative of different functional requirements of the mammary gland or simply differences in hormonal regulation is uncertain at present.

The competitive PCR analysis of the VEGF receptors has proven to be an extremely useful method of quantitatively measuring the receptors in tissue samples. Unfortunately, it is not yet possible to quantitate the amount of receptor protein by Western blotting because antibodies are not available which are sufficiently sensitive in detecting the receptors from tissue lysates. Although the PCR methods (including for VEGF) are measuring mRNA quantity, it is assumed that this is a fair representation of differential protein level in the various tumors. It is certainly possible that there are differences in mRNA stability among tissue samples which could result in inconsistencies between RNA and protein expression levels, but presently there are not data to support this possibility. We are still hopeful that we or others will generate antibodies which will allow a clean and sensitive measure of VEGF and VEGF receptor protein and we are actively testing our new anti-receptor antibodies.

The data on the receptor expression very closely matches that from the VEGF expression data, in that, where a noted increase in VEGF expression was detected, similar increases were seen for the receptors. Most notably when comparing normal mammary

with and without estrogen, there is a marked increase in the expression of Flk. As with VEGF expression, estrogen minimally affects receptor expression in the tumors. There may be a down regulation of Flt in MXTOVEX tumors with estrogen. However, these data are representative of only two tumors and more samples must be included. Statistical analysis will be applied to determine if there are correlations between isoform and receptor expression. Furthermore, continued experiments are in progress to determine whether both receptors respond identically to the presence of estrogen.

Our data obtained on the morphological changes in the normal mammary gland with estrogen are surprising and exciting. These data demonstrate a morphological influence which has previously been attributed to a direct action of VEGF (61). As described in the conclusions, whether the modifications in the vascular endothelium (fenestrations and fused, enlarged caveolae) are the direct result of estrogen or the result of estrogen increasing VEGF is presently not clear and will require further study. The main physiological result of endothelial fenestration and vesicle fusion is an increase in vascular permeability. Practically, this conceivably may allow additional treatment regiments which can preferentially increase the vascular permeability of the blood vessels in the surrounding mammary gland which feed the tumor. Obviously, a local increase in vascular permeability may allow the delivery of more chemotherapeutic agent or other anti-tumor agents which classically have difficulties getting out of the vasculature and into the tumor (e.g. antibodies, liposomes, etc.). At a more cell biological level, these data represent a positive correlation between increased VEGF expression and endothelial fenestration which can be experimentally manipulated. This new model may allow further studies on the mechanism by which fenestrations are formed and maintained and their role in microvascular permeability.

(7) CONCLUSIONS

It is absolutely necessary that our studies include the normal mammary gland in the analysis of VEGF and the receptors. Although the focus of our research is the tumor vasculature, tumor vessels are derived from the normal mammary gland in our models. We must therefore characterize the VEGF expression and more importantly the receptor expression to understand how this is modified in the tumor and affected by factors which

influence mammary biology, namely estrogen. We had hypothesized that estrogen would increase VEGF expression in the mammary gland as it had done in other estrogen responsive organs. Although VEGF can induce the expression of the receptors on neovasculature, it was not certain that the amounts increased in the mammary gland would result in an upregulation of the VEGF receptors in non-proliferating vessels. There are increases in receptor expression in normal mammary and MDA-MB-231 tumors with estrogen. We can not yet determine whether the increased expression of Flk and Flt in response to estrogen is the direct result of hormonal up-regulation or the result of increased VEGF expression (in response to estrogen). Further experimentation will be necessary to distinguish between these two possibilities. We may be able to determine which mechanism is responsible by injecting anti-VEGF antibodies into the animal prior to receptor analysis. In summary, we have examined VEGF and VEGF receptor expression and vascular morphology in normal mammary and breast cancers with and without estrogen. There appears to be a positive correlation with estrogen presence, increased VEGF/VEGF receptor expression, and VEGF-associated vascular morphologies (fenestrated endothelium) in the mammary gland.

Our data may be the first observation that estrogen differentially regulates the VEGF isoforms. This is the type of result we were hoping to find when we initated this project, because it may provide insight into whether there are functional differences among the isoforms. Oue Statement of Work for the second year has two tasks: receptor localization by immunohistochemistry and characterization of changes in tumor vascular morphology by electron microscopy. We have already begun these studies and do not foresee insurmountable problems in completing these tasks. Additionally, we will continue studies to test the usefulness of the competitive PCR measurement of the receptors as a prognostic indicator of microvessel density.

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(9) APPENDICES

Figure Legends

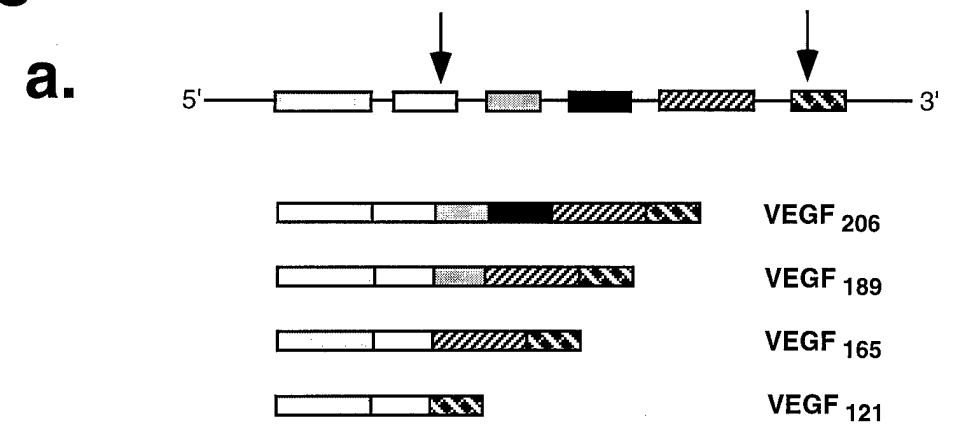
1. VEGF isoform expression in normal mammary tissue and breast cancers in animals implanted with estrogen pellets ($+E_2$) or with placebo pellets ($-E_2$). **a.** A schematic of the VEGF gene with the results splice varients. The arrows show the sequence which the primers recognize. **b.** A representative gel demonstrating the effects of estrogen on VEGF isoform expression in the various tissue samples. **c.** Densitometric analysis of data represented in b. Bars represent average and SD of 2-6 samples.

2. VEGF receptor expression in normal mammary tissue and breast cancers in animals implanted with estrogen pellets ($+E_2$) or with placebo pellets ($-E_2$). **a.** Schematic representation of the receptors and the competitive templates used in the PCR analysis of Flk and Flt. **b.** A representative example of the data and graph used to determine amounts of receptor expression. **c.** Densitometric analysis of data represented in b. Bars represent average and SEM of 2-4 samples. mam, mammary; ovex, MXT-OVEX tumor; MDA, MDA-MB-231 tumor; N.D., not determined.

3. Electron micrograph of normal mammary gland from ovariectomixed (OVX) animal implanted with a placebo pellet (a). Note the numerous caveolae, most of which are not fused nor enlarged. In contrast, the normal mammary gland from an OVX animal implanted with an estrogen pellet demonstrates microvascular endothelium which is extensively fenestrated (arrowheads) and attenuated (b). Additional morphological findings were increased size and fusion of the caveolae (V) (c). The vascular morphologies found in the mammary gland in the presence of estrogen are identical to those which have been observed in tumor vessels and other vessels as a direct result of exposure to VEGF. Bars: a. 1 μm, b. & c. 600 nm.

Animal Use Reporting Form 52-R

Figure 1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3'
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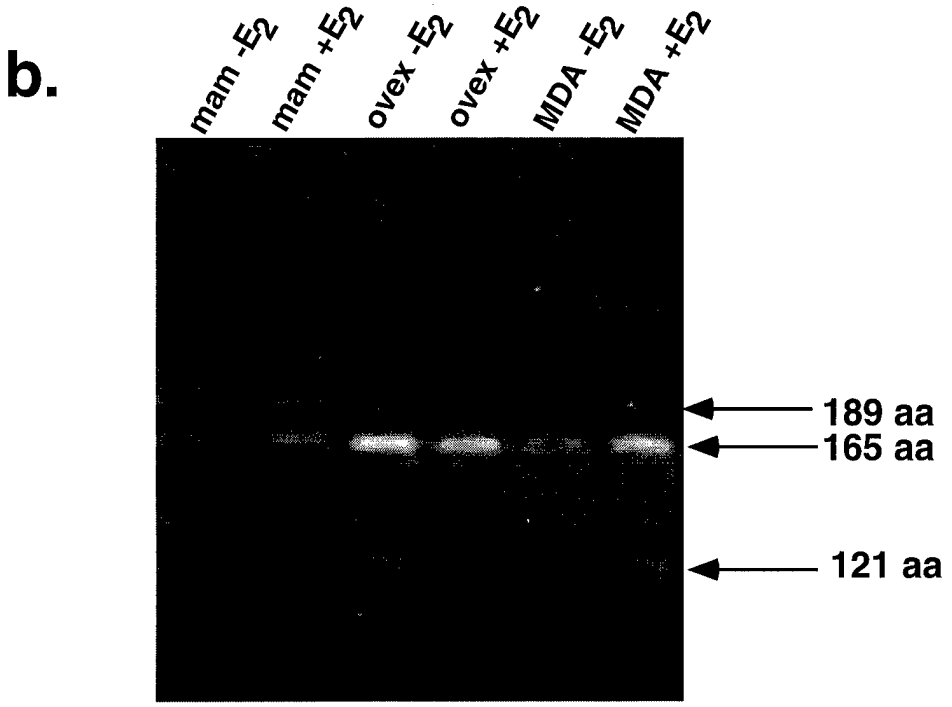
VEGF 189
VEGF 165
VEGF 121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MDA -E2
MDA +E2
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165 aa
121 aa

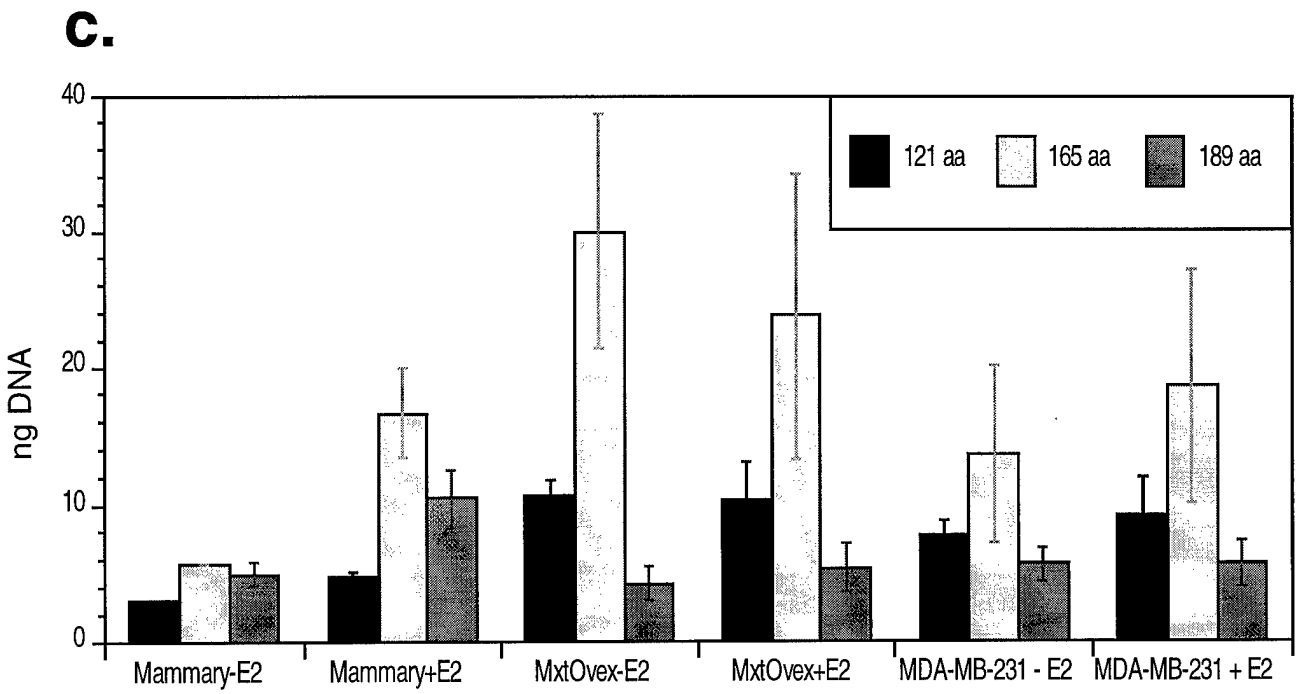
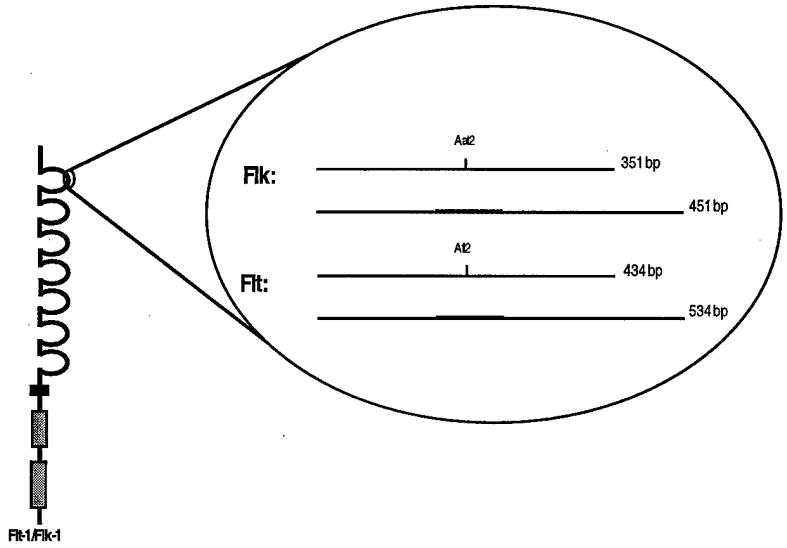

c.
40
30
20
10
0
ng DNA
121 aa
165 aa
189 aa
Mammary-E2
Mammary+E2
MxtOvex-E2
MxtOvex+E2
MDA-MB-231 - E2
MDA-MB-231 + E2

Figure 2

a.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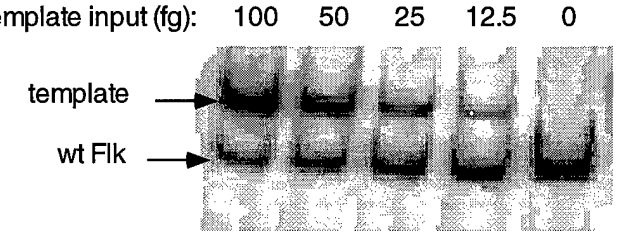
451 bp
Flt:
At2
434 bp
534 bp
Flt-1/Flk-1

b.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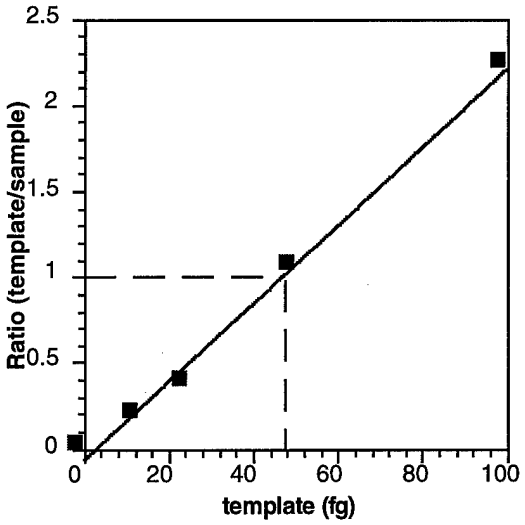
template
wt Flk


Ratio (template/sample)
template (fg)
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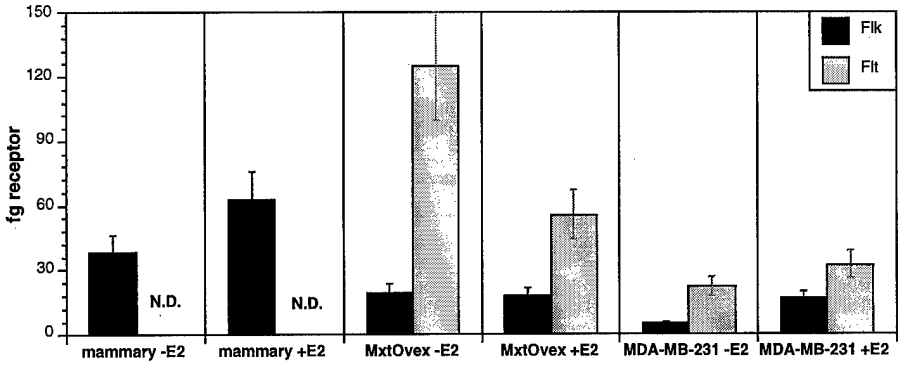
1
1.5
2
2.5
0
20
40
60
80
100

c.


fg receptor
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Flt
N.D.
N.D.
mammary -E2
mammary +E2
MxtOvex -E2
MxtOvex +E2
MDA-MB-231 -E2
MDA-MB-231 +E2

Figure 3